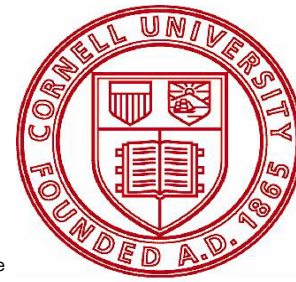


# Detection of Bacterial Speck, *Pseudomonas syringae* pv. *tomato* using Real-Time PCR

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## Background

*Pseudomonas syringae* pv. *tomato* (Pst) is the causal organism of bacterial speck on tomato plants. It is a Gram-negative bacterium that is characterized by the fluorescent color produced when cultured on King's B growth medium (Figure 1). The specific strain of *Pseudomonas syringae* used was Pst A9. Exposed plants develop small, necrotic lesions on their fruit, stem, and/or leaves (Figures 2-4.) In serious cases, the bacteria can overcome and kill the plant. The focus of this project was to use real-time polymerase chain reaction (PCR) technology to detect and quantify the amount of Pst present on tomato leaf samples following inoculation with the pathogen. Quantification on plants treated with Actigard, Bioyield and Cuprofix, Pst control products, was also examined to see if treatments actually reduce the amount of bacterial growth. The purpose of this experiment was to further understand the correlation between bacterial growth and visible disease symptoms. Previous research has shown that the two are not always directly related (1). This experiment also served to demonstrate the effectiveness of various Pst control products that attack the bacteria in different ways; Cuprofix kills the bacteria while Bioyield and Actigard induce the activation of plant defense mechanisms to stop bacterial colonization. There are various advantages and disadvantages to each.

## Materials and Methods

DNA was extracted from leaf samples of Pst A9 inoculated tomato plants grown in the field. The samples were collected 12 hours prior to inoculation, 12 hours after, 36 hours after, 60 hours after and when there were visible speck symptoms. DNA was extracted using a TissueLyser system to grind the leaf samples. A standard extraction protocol was followed from a Qiagen DNeasy handbook, Qiagen, Inc. (Madison, WI).

To determine the amount of Pst on the leaves, real-time PCR was performed three times, on each of the three replicates of each treatment (Figures 6 and 7). Real-time PCR amplifies DNA based on the amount present in samples, so samples with the highest amount of Pst were expected to amplify at an earlier cycle. Amplification was compared to a standard real-time curve that used 10-fold dilutions of Pst DNA (Figure 8).



Figure 1. *Pseudomonas syringae* on King's B.



Figure 2. Speck lesions on developed fruit.



Figure 3. Speck lesions on a young plant.

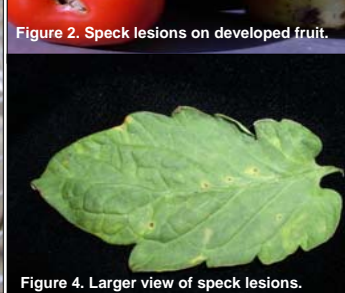


Figure 4. Larger view of speck lesions.

## Results and Discussion

The data from real-time PCR amplification demonstrated a correlation between visible speck symptoms and the amount of bacteria on the plant (Figure 9+10). Variation was present among the five different types of Pst treatments used on the tomato plants. The plants treated with Actigard+Bioyield had the least amount of bacteria on their leaves when the samples were collected from plants that had visible symptoms.

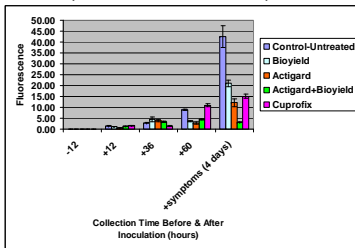


Figure 9. Real-time PCR data collected from 75 DNA extractions. Each treatment represents the average of three repetitions. Collection times spanned over approximately a four-day period with plants displaying visible speck symptoms collected on "+symptoms" time point.

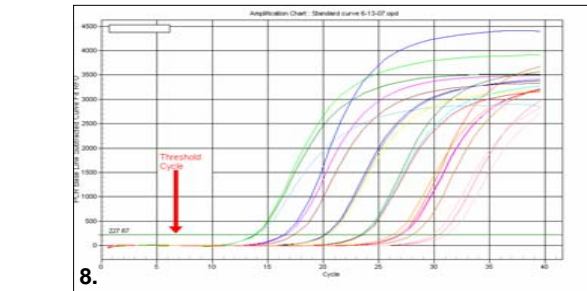
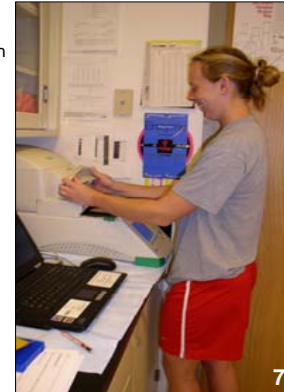


Figure 5. Nanodrop spectrophotometer used to determine the concentration of extracted DNA from each leaf sample. Figure 6. Preparing DNA samples for real-time PCR. Figure 7. Loading the real-time PCR machine. Figure 8. Standard Pst A9 curve showing 10-fold dilutions of Pst A9, 100 ng/2.5 µl to 0.01 ng/2.5 µl.

Disease symptom data (Figure 10) showed that plants treated with Cuprofix, Actigard and Actigard+Bioyield had the lowest number of visible speck lesions on their leaves. The disease symptom data from 2006 mimicked the data collected in this experiment and suggest that visible speck symptoms directly correlate to the actual amount of bacteria present on the plants.

Treatment	Lesion # 7/24*	Lesion # 7/28*
Control	13.3 a	79.6 a
BioYield	10.5 b	59.1 b
Cuprofix 40	7.9 c	32.5 c
Actigard 50 WG	5.8 c	24.8 c
Actigard + BioYield	6.7 c	25.7 c

Figure 10. 2006 disease data. Lesion number represents the mean number of lesions counted on 20 leaflets per plot. The samples from 2006 were the same ones used in this experiment.



Real-time PCR performed in this study utilized the SYBR green reporter dye system. The dye, buffer and *Taq* DNA polymerase were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA). To prepare for real time PCR, a specific master mix was added to DNA from each sample. The master mix was composed of SYBR green, two primers for Pst and nuclease-free H<sub>2</sub>O. For Pst A9, the two primers used were HrpZF1 and MM5R(3). Each primer was at a concentration of 5 µM. The master mix was always made for n+4 samples (to ensure a sufficient amount) and an amount of 16 µl of mix was added to DNA and nuclease-free water. To determine the amount of DNA to add to each sample, the samples were analyzed using a Nanodrop system; a spectrophotometer that determines DNA concentration in ng/µl for a 2 µl drop of each sample (Figure 5). Results from the Nanodrop were verified using gel electrophoresis and varying amounts of DNA were added so that each well contained approximately 160 ng/µl of DNA. Nuclease-free H<sub>2</sub>O was added to each well so that the total volume of DNA, nuclease-free water and master mix was 24 µl.

## Future Work

Real-time PCR has become a widely used technology in quantifying pathogen growth on plants. Research has shown that disease symptoms do not always correlate with colonization, making real-time PCR an important step in overall diagnosis. The quantification of Pst A9 on the tomato leaf samples in this experiment specifically demonstrated the variability in different Pst control products. Data from this experiment and others point to the similar effectiveness of more environmentally-friendly plant activators such as Actigard; a product that induces plant defense mechanisms rather than directly attacking the bacteria (2). The use of plant activators could prevent the occurrence of copper-resistant strains of Pst. Quantifying bacterial growth on a plant with real time PCR provides data that enable the development of appropriate disease control strategies.

## Acknowledgements

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